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13. ABSTRACT (Maximum 200 Words)

Oxidative stress may play a role in human oncogenesis, including breast cancer. The mitochondria are most frequent sources of reactive oxygen species (ROS) responsible for most oxidative stress. This project evaluates the role of mitochondrial abnormalities in oxidative stress in breast cancer development. An advanced cre-LoxP gene activation strategy will be used to over-express mutant mitochondrial complex II subunits in the mammary glands of transgenic mice. These mutations are responsible for elevated levels of ROS in the cells. The transgenic mice will be characterized in terms of mitochondrial functions, ROS productions and oncogenesis in the mammary glands. The effects of oxidative stress in other transgenic mouse models of breast cancer will be evaluated. Bi- or tri-transgenic mice will be generated by crossing and analyzed in terms of their breast cancer development, in the presence or absence of mitochondrial mutant gene and hence oxidative stress. This study should provide significant information regarding the role of oxidative stress in breast cancer development and progression, and insights on whether antioxidants are beneficial in prevention and treatment of such important cancer in women.

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INTRODUCTION

Oxidative stress has been postulated to contribute to numerous human diseases, including aging, neurodegeneration, cardiovascular disease, and cancers (1,2). Since most studies were conducted in epidemiological studies, the experimental proof for such a causative effect has been lacking. The overall goal of this project is to address this question in transgenic mouse modeling, focusing on breast cancer.

Numerous mutations with various mitochondrial components have been demonstrated to cause increases in reactive oxygen species (ROS), the ultimate mediators of oxidative stress. In particular, mutations in the subunits of the mitochondrial complex II have been demonstrated to produce structural abnormalities, impairing energy production and electron transport, and generation of ROS (3). To address the roles of mitochondrial structural abnormalities and ROS in breast cancer development and progression, an advanced strategy will be implemented to over-express a subunit of the mitochondrial complex II in the mammary glands of transgenic mice. The effects of such mitochondrial abnormalities and elevation of oxidative stress will be evaluated in these animals in terms of breast cancer development under normal and pre-disposed conditions. The first year of this project focuses on the assembly of the components of this transgenic strategy.

BODY

The Cre-LoxP Gene Activation Strategy (4)

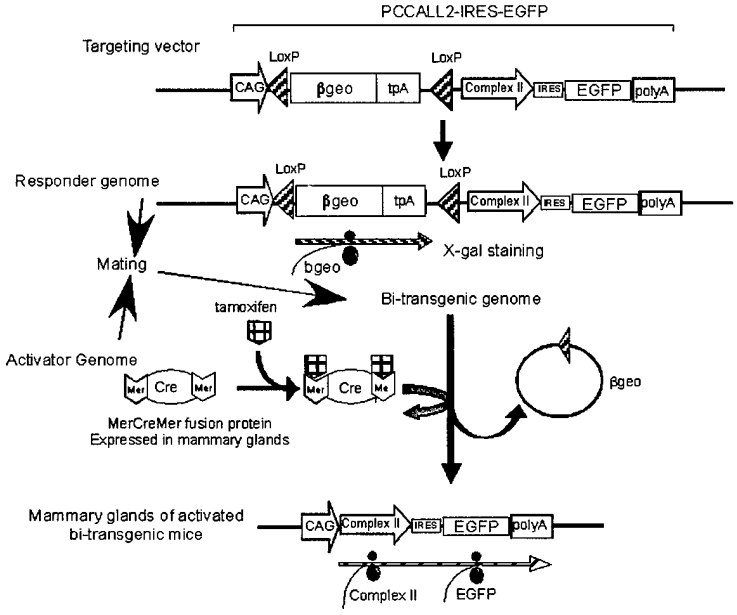

Figure 1. Cre-LoxP Gene Activation

The strategy consists of two components: the activator and responder transgenes (Figure 1). The activator harbors a Cre transgene directed by a mammary gland specific promoter, such as whey acidic protein gene or MMTV promoter. The responder harbors a bicistronic transgene directed a strong actin promoter. Independently, these two transgenes will not produce any mutant complex II subunits. However, when they are present in the same mice, expression of the Cre recombinase will induce a recombination of the responder gene, thereby placing the complex II mutant directly under the regulation of the strong actin promoter and high level expression of the mutant gene. Since the activator gene will only be expressed in the mammary glands, only this organ will be subjected to the oxidative effects and consequences of mitochondrial complex II mutant gene expression.

Task 1. To construct and characterize mutant mice

a. Construction of responder lines

For the past year, we have focused on designing and constructing mutant genes coding for mouse complex II subunits that affect the overall structure of this mitochondrial complex. The elucidation of the atomic structure of this complex (9) has provided the basis for us to identify specific targets for mutations that will have profound effects on its structural stability and function (Figure 2). The first mutation is a valine (V) to glutamic acid (E) change at position 69 on the subunit C (V69E). This residue forms part of the quinone pocket for complex II. It provides stability for the quinone to acquire the electrons and to transport them to complex III. Disruption of the quinone pocket affects this electron transport mechanism and might result in unstable electrons that are easily transferred to molecular oxygen and generating of reactive oxygen species. The second mutation is a histidine (H) to leucine (L) change at position 102 on subunit D (H102L). This histidine is located at the second helix of subunit D and is a ligand for heme b. Disruption of this ligand will affect the stability of complex II. Heme b could serve as an electron reservoir for the electron transport chain within this complex. Disruption of its structure will produce unstable electrons at the quinone pocket, thereby resulting in ROS generation. Further, heme b and subunit D have been postulated to be involved in oxygen sensing mechanism. Disruption of its stability might lead to abnormal oxygen sensing and elicitation of hypoxia responses, in the absence of any real hypoxia. One key response for hypoxia is cell proliferation and tissue vascularization (5). Hence, beside ROS generation, the H102L mutation will also be able to address the possibility of abnormal oxygen sensing as a mechanism for tumorigenesis.

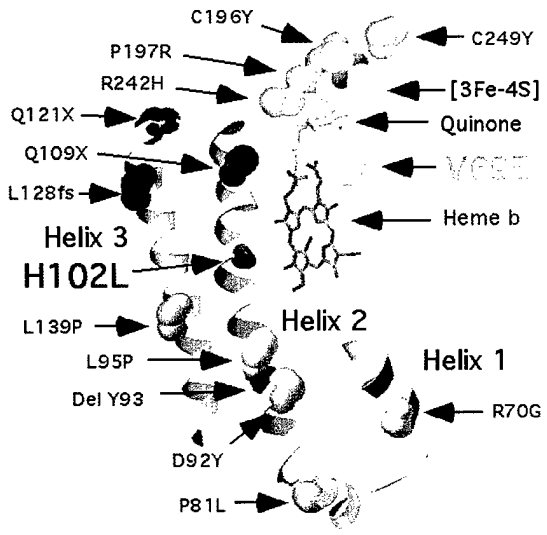

Figure 2. Mutations of Complex II

Using the standard site-directed mutagenesis techniques, we had generated these two specific mutations in the coding sequences for subunits C and D. They were designated as mSdhC-V69E and mSdhD-H102L respectively. They were then inserted into the targeting vector, PCCALL2-IRES-EGFP, as described in Figure 1. The entire expression cassettes were then excised, and used in transgenic mouse construction. Four sessions of microinjection were performed with the mSdhC-V69E construct, resulting in approximately 65 liveborns. Of these, 16 founder animals were obtained. For the mSdhD-H102L, two microinjection sessions were performed, resulting in 24 liveborns. Of these, 6 founder animals were obtained.

To identify transgenic mouse founders that harbor functional transgenes, tail tissues were processed for detection of βgeo (= lacZ gene coding for β-galactosidase) expression. Based on the design of the targeting vector, βgeo should be expressed without any activation by the Cre recombinase action. Upon activation, βgeo sequence will be excised by the Cre recombinase while the complex II-EGFP expression cassette will be re-positioned directly after the actin promoter. We surmise that this initial testing of the actin promoter is essential because if this promoter is incapable of directing the transgene expression (perhaps due to inhibition by the flanking sequences of integration site), it will not be useful in directing the complex II-EGFP expression after the recombination and activation. Of the 16 founder lines for the mSdhC-V69E construct, 3 showed consistent β-galactosidase activities in their tail tissues and 3 showed low levels of enzyme activities. These

transgenic lines were further characterized with detailed β-galactosidase activities in various internal organs (e.g. Figure 3). The mSdhD-H102L transgenic lines are currently being analyzed similarly.

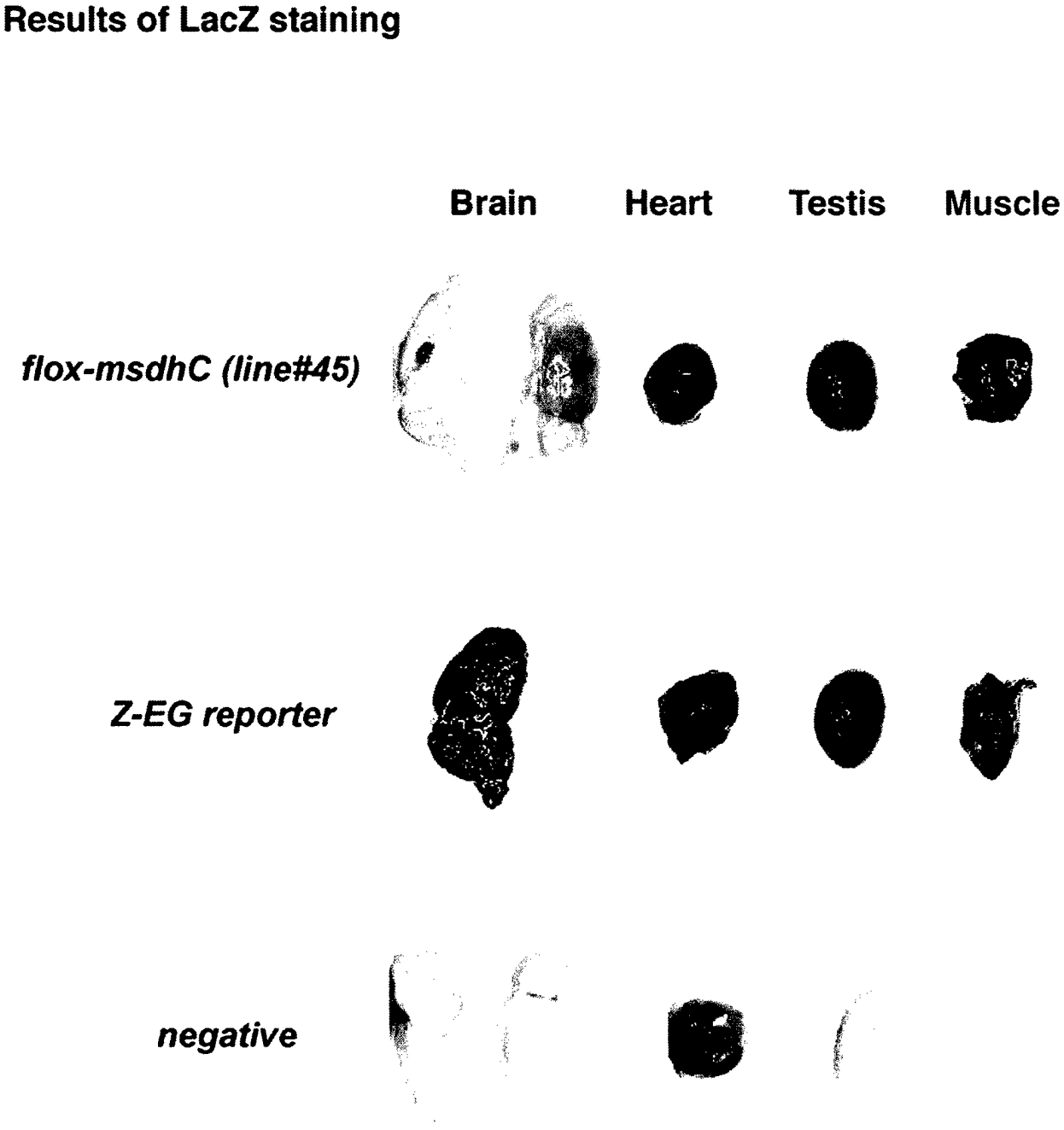

Figure 3. Detection of β-galactosidase activities in tissues of transgenic mice. Z-EG reporter is a positive reference line. Flox-mSdhC is line #45 harboring a mSdhC-V69E transgene. Negative control is a non-transgenic mouse.

b. Construction and acquisition of activator lines

Currently, there are numerous available transgenic mouse lines from established repositories, such as the Jackson Laboratory and the Mouse Models of Human Cancers Consortium at the National Cancer Institute – Frederick. Two particular Cre recombinase lines should be most suitable for the present strategy. They are described below.

1. WAP-Cre line (7). Mice from this transgenic line harbor a Cre recombinase transgene directed by the whey acidic protein (WAP) gene promoter. Cre is specifically expressed in the secretory epithelium of the mammary gland in this strain.

2. WAP-rtTA-Cre (8). Mice from this transgenic line harbor an inducible Cre recombinase directed by the whey acidic protein (WAP) promoter. Cre recombinase expression is localized to the mammary gland and is controlled by the administration of doxycycline. This line should be useful in establishing an inducible system to activate the Cre recombinase activities, thereby the recombination of the targeted complex II mutant – EGFP responder transgene. This inducible system will be tested against those utilizing tamoxifen as an inducer molecule. If it works with similar or better efficiency, we will adopt this system in lieu of the tamoxifen one, originally proposed.

Currently, we have just completed the Material Transfer Agreements with the Mouse Models of Human Cancers Consortium and should be expecting these mice within a few weeks. Additional reference lines have been obtained and integrated into our transgenic mouse colonies. They include the following strains.

3. CAG-Cre (6). Mice from this transgenic line harbor a Cre recombinase directed by the actin promoter. Expression of the Cre recombinase is widespread. We have obtained this line from Dr. Corrine Lobe, University of Toronto. This line will be used as a reference line for the present studies.

4. CAG-Cre^{TM} (4). Mice from this transgenic line harbor an inducible Cre recombinase transgene directed by the actin promoter. Expression of the Cre recombinase is widespread. However, it remains inactive in the cytoplasm of the cells. It can be translocated to the nuclei upon tamoxifen administration. Once it enters the nuclei, it can then cleave the floxed responder gene, thereby activating the complex II mutant. We have already obtained this line from Dr. Corrine Lobe, University of Toronto. This line will be used as an inducible reference line for the present studies.

FUTURE DIRECTIONS

We believe we have made significant progress within the first year of this project and have implemented all the necessary steps to accomplish the stated objective in evaluating the role of mitochondrial structural integrity and oxidative stress in mammary oncogenesis. Since all the Cre transgenic lines have been demonstrated to be capable of cleaving sequences flanked by two loxP sites, the immediate task for this project is to identify a suitable responder line(s) capable of being activated in the mammary glands by these Cre recombinase lines in bi-transgenic mice. Currently, we are exploring several avenues to achieve this objective. In addition to identifying responder lines capable of expressing the β-geo gene in the mammary glands, other approaches, such as explanted cell from

responder lines and activation of responder genes in cultured cells and crossing with reference lines, are being explored. We are confident that once identified, these responder lines will be most useful in crossing with the above mammary gland specific Cre recombinase lines for the proposed studies.

KEY RESEARCH ACCOMPLISHMENTS

- Construction of complex II mutant subunit genes by site-directed mutagenesis
- Construction of transgenic mouse lines harboring mutant complex II – EGFP expression cassettes
- Completed the MTA processes in obtaining established transgenic lines harboring mammary gland specific Cre recombinases

REPORTING OUTCOMES

None.

CONCLUSION

We have made good progress in establishing the components essential for studies proposed in the original application. The availabilities of these components will facilitate our studies to evaluate the role of mitochondrial structural integrity and oxidative stress in breast cancer development.

SO WHAT

Oxidative stress has been implicated in the etiologies of numerous human diseases. Successful implementation of the proposed research will provide critical insights on its role(s) in breast cancer. The availability of experimental animal models of breast cancer, pertaining to oxidative stress, will be important in understanding the disease mechanism, potential prevention and therapeutic intervention for this devastating human cancer.

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